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Abstract

In the current contracting period, we utilized specific and potent inhibitors of different caspases to determine the most effective means of preventing SM toxicity in vitro and vesication in vivo. We obtained tetrapeptide inhibitors to each of the caspases and treated KC for 30 min prior to SM exposure. In addition to determining total SM toxicity, we delineated the specific pathway for the activation of each of the caspases. When primary KC were treated for each of the caspase inhibitors, prior to SM exposure and assayed for activation of each caspase, IETD and LEHD (caspase-8 and caspase-9 inhibitors) were the most effective caspase inhibitors for human KCs in culture. These worked more effectively than the pan-caspase inhibitor, ZVAD. We have now utilized these inhibitors as compounds for testing in the in vivo human skin graft for their ability to block SM vesication. Primary human KC were used to establish a histologically and immunocytochemically normal epidermis grafted onto the back of nude mice. To test the effects of the peptide inhibitors of caspases on apoptosis and vesication in intact human epidermis, normal human skin was grafted onto the back of athymic mice, and 6-8 weeks after grafting, we inhibited the activity of caspases in vivo by topical application of the inhibitors at the graft site 30 min prior to SM exposure. These human grafts were then exposed to SM by vapor cup method, and showed a vesication response, utilizing an end point of micro or macro blisters. Frozen and fixed sections derived from graft sites of these animals were analyzed for apoptosis markers. Histological analysis of SM-exposed animals grafted with primary KC showed that SM microvesication can be reduced by topical application of zVAD-fmk. While there was no difference in the DMSO (vehicle)-treated and ZFA-treated control skin grafts, there was a notable decrease in the amount of microvesication in grafts treated with zVAD-fmk. Sections from SM-exposed human skin grafts were also subjected to analysis for markers of in vivo apoptosis, including immuno-fluorescence microscopy with antibodies to active caspases-3, -8, and -9, and detection of apoptotic DNA breaks by in situ TUNEL staining. Apoptotic cells were concentrated in the areas of microvesication. SM induced apoptotic DNA breaks primarily in the basal cells of DMSO-treated control human skin grafts, but was attenuated in the grafts treated with zVADfmk. Pretreatment of the skin graftswithindividualcaspases8or9peptideinhibitorswerelesseffectiveinmodulatingthisresponseSMactivatesbothadeathreceptorandmitochondrial

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FOREWORD

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1. OVERVIEW

During the previous contracting periods we focused primarily on the *in vitro* effects of sulfur mustard (bis-(2-chloroethyl) sulfide; SM), the highly reactive vesicant agent used in 1988/89 in the Iraq/Iran conflict and presumably in the Gulf War, which is potently cytotoxic to cultured keratinocytes. We showed in previous technical reports and published studies that SM induces both terminal differentiation and apoptosis in human keratinocytes (KC; (Rosenthal et al., 1998c), while human dermal fibroblasts contribute to the vesication response by releasing degradative cytosolic components after a PARP-dependent SM-induced necrosis (Rosenthal et al., 2001). In humans, SM produces severe skin blisters *via* its ability to induce the death and detachment of the basal cells of the epidermis from the basal lamina [Papirmeister, 1991 #799; Meier, 1984 #804; Gross, 1988 #803; Petrali, 1990 #802; Smith, 1990 #805; Smith, 1991 #801].

2. Completion and Publicatin of CaM1 inhibitor Studies

2.1 Introduction

We have also previously reported that SM cytotoxicity is related to changes in intracellular Ca^{2+} (Ca_i) and calmodulin (CaM) (Mol and Smith, 1996; Ray et al., 1995; Ray et al., 1993; Rosenthal et al., 1998b). During the present contracting period, we have been able to complete these studies and have recently published these findings in the Journal "*Toxicology*":

(Simbulan-Rosenthal CM, Ray R, Benton B, Soeda E, Daher A, Anderson D, Smith WJ, Rosenthal DS. Calmodulin mediates sulfur mustard toxicity in human keratinocytes. Toxicology. 2006 Oct 3;227(1-2):21-35. Epub 2006 Jul 13. PMID: 169354)

2.2 Materials and Methods

Cells, Plasmids, and Transfection. Primary human KC were prepared from neonatal foreskins and maintained in serum-free medium (SFM) supplemented with human recombinant EGF and bovine pituitary extract (Life Technologies). A replication-deficient recombinant retroviral construct expressing *CaM1* AS was constructed by subcloning into the retroviral vector pLXSN. Correct insertion of CaM1 AS cDNA in the pLXSN vector was confirmed by restriction enzyme digestion mapping, as well as by DNA sequencing. This retroviral construct, as well as empty vector pLXSN were then packaged and propagated in the amphotropic producer cell line øNX (provided by Gary Nolan, Stanford University) by transfection using Lipofectamine 2000 (Life Technologies) according to manufacturer's protocols; viral supernatants were derived, filtered, and used to transduce KC. Retrovirus-infected cells were selected for 10 days in G418, and resistant colonies were pooled from each transduction. Immunoblot analysis was performed on expanded pooled cells to confirm loss of CaM1 expression.

For exposure to SM, cells were grown to 60-80% confluency, and then exposed to SM diluted in SFM to final concentrations of 100, 200, or 300 μM, with or without pretreatment with CaM inhibitors. Media was not changed for the duration of the

experiments. At indicated time points after SM exposure, cells were harvested for further analyses.

Chemicals. SM (*bis*-(2-chloroethyl) sulfide; >98% purity) was obtained from the US Army Edgewood Research, Development and Engineering Center. The CaM antagonist W-13 (N- (4-Aminobutyl)-5-chloro-2-naphthalenesulfonamide, HCl), its less active structural analogue W-12 (N- (4-Aminobutyl)-2-naphthalenesulfonamide, HCl), the calcineurin inhibitor cyclosporin A (CsA), as well as CaMKII inhibitors KN62 and KN93 were purchased from Sigma-Aldrich (St. Louis, MO).

Reverse transcription-polymerase chain reaction (RT-PCR). Unique oligonucleotide primer pairs for CaM1, CaM2, CaM3, and CLP mRNA were designed and prepared. RT-PCR was performed with total RNA, purified from cell pellets with Trizol Reagent (Life Technologies), utilizing a Perkin Elmer Gene Amp EZ rTth RNA PCR kit according to manufacturer's specifications. After RNA was transcribed at 65^{o} C for 40 min, DNA was amplified by an initial incubation at 95^{o} C for 2 min, followed by 30 cycles of 95^{o} C for 1 min, 60^{o} C for 1.5 min, and 65^{o} C for 0.5 min, and a final extension at 70^{o} C for 22 min. PCR products were then separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Fluorometric assay of caspase-3 activity. Cytosolic extracts were derived from pooled floating and attached cells and subjected to fluorometric caspase-3 activity assays using fluorescent tetrapeptide substrate specific for caspases-3 (Ac-DEVD-aminomethylcoumarin (AMC), BioMol) as previously described (Simbulan-Rosenthal et al., 2002). Free AMC, generated as a result of cleavage of the aspartate-AMC bond, was monitored over 30 min with a Wallac Victor^{3}V fluorometer (Perkin Elmer) at

excitation and emission wavelengths of 360 and 460 nm, respectively. The emission from each sample was plotted against time, and linear regression analysis of the initial velocity (slope) for each curve yielded the activity.

Immunoblot analysis. SDS-PAGE and transfer of separated proteins to nitrocellulose membranes were performed according to standard protocols. Proteins were measured (DCA protein assay; BioRad, Hercules, CA) and Ponceau S (0.1%) staining of membranes was performed to verify equal loading and transfer of proteins. Membranes were then incubated with antibodies to CaM (1:1000; Sigma), the p17 subunit of caspase-3 (1:200; Santa Cruz Biotech), procaspase-7 (1:1000; PharMingen), procaspase-8 (1:1000; PharMingen), procaspase-9 (1:1000; Cell Signaling), caspase-6 (1:1000; Trevigen), PARP (1:1000; PharMingen), Bad (1:1000; NEB), or Phospho-Bad (1:1000; NEB). Immune complexes were detected by subsequent incubation with appropriate horseradish peroxidase–conjugated antibodies to mouse or rabbit IgG (1:3000) and enhanced chemiluminescence (Pierce, Rockford, IL). Immunoblots were sequentially stripped of antibodies and reprobed with additional antibodies to compare different proteins from the same filter.

Hoechst Staining. For detection of apoptotic nuclear morphology, cells were fixed for 10 min in PBS containing 4% formalin, washed with PBS, and stained with Hoechst 33258 (24 mg/ml) in PBS containing 80% glycerol. Nuclear morphology was observed with an Olympus fluorescence microscope equipped with a cooled CCD camera.

Statistical Analysis. For caspase-3 activity assays, data were compared using 2-way ANOVA tests for significance; p values of <0.05 were considered statistically

significant. The results are representative of at least 3 independent experiments with reproducible results.

2.3 Results and Discussion

The exquisite sensitivity of the skin to SM vesication is due in part to the unique response of KC to Ca^{2+}-signaling pathways. While many CaM-dependent processes may be dependent on the availability of Ca^{2+}, we showed in this published article that SM-induced death and detachment of epidermal basal cells from the basal lamina involves changes in intracellular levels of both Ca^{2+} and CaM (Gross et al., 1988; Meier et al., 1984; Papirmeister et al., 1991; Petrou et al., 1990; Rosenthal et al., 1998c; Smith et al., 1990; Smith et al., 1991; Smulson, 1990). Previous evidence for regulation of CaM activity by modulation of CaM levels has been presented for a number of cell and tissue types in both normal and disease states (Geoffrey Burwell and Dangerfield, 2003; Hulvershorn et al., 2001; Krishnaraju et al., 1991; Lin et al., 2000; Lowe et al., 2002; Michelhaugh and Gnegy, 2000; Toutenhoofd and Strehler, 2000; Wollina et al., 1987). In the skin, increased CaM levels are associated with, and may actually induce hyperproliferative states, including psoriasis (Kaur et al., 1991; Rasmusen and Means, 1990; Rasmussen and Means, 1992; van Erp and van de Kerkhof, 1987; van Erp and van de Kerkhof, 1988).

Basal cell death of KC is primarily due to apoptosis at the doses tested (100- 300 µM of SM), contributing to SM vesication (Rosenthal et al., 1998c). Clearly, meaningful studies using cell culture models should include SM concentrations that yield alkylation levels similar to those that produce injuries in humans. In human keratinocytes and lymphocytes, doses between 100 µM and 1 mm were shown to induce $2x10^6$- $2x10^7$ SM

alkylations/genome resulting in cell death (Mol et al., 1989). In human skin injury, similar levels of alkylation (10^6- 10^7 adducts/genome; 1.0- 2.5 µg SM / cm^2) resulted in vesication (Renshaw, 1946). In contrast, lower levels of SM alkylation did not induce cytotoxicity in a variety of mammalian cells (2 x10^2- 2x 10^4 adducts/genome; 0.01- 1 µM SM dose; (Fox and Scott, 1980) or overt tissue injury in human skin (2 x 10^3 adducts/genome; .01 µg SM/cm^2; (Illig et al., 1979). We therefore believe that the doses used in the current study are reasonable cell culture equivalents of vesicating doses.

The mode of cell death induced by SM has also been examined in other cell types. While SM induces an apoptotic response in HeLa cells (10-100 µM; (Sun et al., 1999), peripheral blood lymphocytes (6 - 300 µM; (Meier and Millard, 1998), KC (50-300 µM; (Rosenthal et al., 1998c; Stoppler et al., 1998), and endothelial cells (<250 µM; (Dabrowska et al., 1996), a time- and dose- dependent shift to necrosis at higher doses was observed in SM-treated lymphocytes (Meier and Millard, 1998), endothelial cells (>500 µM; (Dabrowska et al., 1996), and HeLa cells (1 mM; (Sun et al., 1999).

Utilizing inhibitors of CaM, published studies, including our own, have demonstrated the importance of Ca^{2+}-CaM complexes in apoptotic cell death (Pan et al., 1996; Rosenthal et al., 1998a; Rosenthal et al., 2000a; Sasaki et al., 1996). Using CaM1 AS in the current study, we have further shown that CaM depletion may block the mitochondrial pathways of SM-induced apoptosis *via* dephosphorylation of Bad. This is consistent with previous studies in which CaM was implicated in the regulation of Bcl-2 family members and thus the mitochondrial pathway of apoptosis. Bcl-2 family members have been shown to interact directly with the CaM-regulated phosphatase calcineurin, which can dephosphorylate (Wang et al., 1997) and regulate the

intracellular localization (Shibasaki et al., 1997) and stability (Haldar et al., 1995) of Bcl-2 family proteins.

In addition to antisense and chemical inhibition, a powerful technique to determine the role of a specific gene product in intact animals as well as in cell cultures derived from them is *via* the use of targeted gene disruption which generate “knockout” animals. Because there are three different human and murine genes that encode CaM, elimination of all CaM protein is problematical. Furthermore, studies in which CaM has been ablated in fungi demonstrate that CaM is essential for survival (Davis et al., 1986; Lu et al., 1993; Sun et al., 1992). Thus, there are currently no CaM knockout higher animals or cell lines in existence, although one group of investigators has performed a functional knockout of CaM in lung epithelium utilizing targeted expression of a CaM-binding peptide (Wang et al., 1995). Two groups of investigators have used a CaM1 AS vector in rat glioblastoma cells (Prostko et al., 1997) as well as in PC-12 cell (Davidkova et al., 1996). Likewise, we found that CaM1 is by far the predominantly expressed gene in primary as well as immortalized KC, and, thus, used a retroviral vector expressing CaM1 AS to substantially reduce the endogenous levels of CaM in KC. Using AS, the present study demonstrates that SM induces CaM-dependent apoptosis in KC mediated by CaM, and involves the activation of caspases-3, -6, -7 and -9. These responses may, in part, explain the death and detachment of basal cells of the epidermis that occurs following exposure to SM. Using a cell culture model in the present study, we describe a potential mechanism for SM-induced KC basal cell death and detachment: apoptosis in KC *via* a CaM-Bad mitochondrial pathway.

KC expressing CaM1 AS exhibited reduced levels of SM-induced PARP cleavage and proteolytic processing of caspases-3, -6, -7, and -9 into their active forms.

In most apoptotic systems, caspase-3, the primary executioner caspase, is proteolytically activated, and in turn cleaves key proteins involved in the structure and integrity of the cell, including PARP, DFF 45, fodrin, gelsolin, receptor-interacting protein (RIP), X-linked inhibitor of apoptosis protein (X-IAP), topoisomerase I, vimentin, Rb, and lamin B (Casciola-Rosen et al., 1996; Nicholson et al., 1995; Slee et al., 2001; Song et al., 1996; Tewari et al., 1995). Caspase-3 is also essential for apoptosis-associated chromatin condensation, DNA fragmentation, and nuclear collapse (Slee et al., 2001).

Our model system has demonstrated a novel role for CaM in SM toxicity. In addition, we have also shown the validity of the use of chemical inhibitors of CaM to block this response. The CaM inhibitor W-13 and its less active structural analogue W-12 were both used at concentrations of 50 μM. At this concentration, the effect of W-13 was specific, since the same concentration of W-12, a less active structural analogue (Hidaka and Tanaka, 1983), did not block Bad phosphorylation, caspase-3 proteolytic processing and activity, and nuclear fragmentation. Similar concentrations of W-13 (15 μg/ml; 43 μM) and W-12 (15 μg/ml; 48 μM) have previously been utilized to demonstrate preferential inhibition of CaM and calcineurin activity by W-13 (Li et al., 2004). Furthermore, we showed that CsA, but not KN62 or KN93, blocked caspase-3 activity following SM exposure, thus supporting the role for a CaM-calcineurin signaling pathway for SM apoptosis. Interestingly, calcineurin has also been shown to contribute to UVB-induced apoptosis in human skin, and, while not examined specifically, the investigators posit that dephosphorylation of Bad may play a role in this system as well (Yarosh et al., 2005).

Since Bad is dephosphorylated following SM exposure, one mechanism for the action of CaM inhibitors is to block the dephosphorylation of Bad, preventing it from complexing with and inactivating Bcl-2, and thereby blocking activation of downstream caspases and cell death. An understanding of the mechanisms for SM vesication will hopefully lead to therapeutic strategies for prevention or treatment of SM toxicity. Although the mechanism for their protection has not been described, CaM inhibitors have already been used successfully in the treatment of both thermal burns and frostbite (Beitner et al., 1991; Beitner et al., 1989) and may prove effective for SM as well, either alone, or in combination with caspase-3 inhibitors. We used antisense oligonucleotide and chemical inhibitors of CaM and have successfully attenuated the apoptotic response in cultured cells, and these inhibitors can be used *in vivo* as well.

While we showed previously that inhibition of the Fas pathway attenuated SM-induced apoptosis, in the current study, we show that knockdown of CaM by CaM-AS also inhibits SM apoptosis, although probably not through a direct interaction with Fas or inhibition of the Fas pathway. Thus, we conclude that the SM-induced apoptotic activities of Fas and CaM are probably through initially separate pathways that exert a cumulative effect on SM apoptosis. Whether this effect is merely additive or synergistic, implying convergence of the two pathways at some point, remains to be determined.

In any case, since CaM inhibitors have been used clinically, and the FADD pathway can be manipulated at the level of a cell surface (Fas/TNF) receptor, these two molecules represent attractive targets for the modulation of the effects of SM in humans. The effects of suppressing the function of the upstream caspases-8 and -9 as well as the downstream central executioner caspase-3 with cell-permeable peptide inhibitors are also currently being investigated. zVAD-fmk, a pan-caspase inhibitor,

appears to be effective in reducing SM toxicity when used in cell culture studies, as well as, in preliminary mouse *in vivo* studies. The use of pharmacological inhibitors of CaM and caspases to study SM pathology, in the context of the whole animal grafted with human skin, is also currently underway, and will offer a better understanding of the mechanism of this damage for human personnel. Inhibition of the CaM-calcineurin-Bad apoptotic pathway by specific pharmacological inhibitors such W-13 or zVAD-fmk may therefore be of therapeutic value in the treatment of or prophylaxis against SM injury in humans.

3. Completion and Publication of Studies on the Effect of retroviral vectors expressing dominant-negative FADD or caspase-9 on human keratinocyte apoptosis

We have reported that SM cytotoxicity induces apoptosis via a Fas-FADD pathway. To determine if this is the mode of cell death induced by other DNA damaging agents, as well as to determine if there was a difference in the response of primary keratinocytes and immortalized keratinocytes, the latter of which are commonly used in published studies, we completed a series of studies and have recently published these findings which reference the current DAMD support in the Journal "*Experimental Dermatology*":

(Daher A, Simbulan-Rosenthal CM, Rosenthal DS. Apoptosis induced by ultraviolet B in HPV-immortalized human keratinocytes requires caspase-9 and is death receptor independent. Exp Dermatol. 2006 Jan;15(1):23-34. PMID: 16364028)

3.1 Introduction

Nonmelanoma skin cancer, the most common of human malignancies, is caused primarily by chronic exposure to solar ultraviolet B (UVB) radiation (280-320 nm) through a series of cellular changes that are not all identified (Cleaver and Crowley, 2002; Stratton, 2001). While the process of photocarcinogenesis is still not fully characterized at the molecular level, genetic alterations, inappropriate or altered differentiation and apoptosis have been shown to play key roles in this process. Others and we have shown that UVB irradiation induces apoptosis and altered differentiation in primary human KC (Jonason et al., 1996; Karen et al., 1999; Mammone et al., 2000; Simbulan-Rosenthal et al., 2005; Simbulan-Rosenthal et al., 2002). This is a potential mechanism for tumor promotion and progression, which allow the preferential clonal expansion of mutant cells, since normal keratinocytes have been shown to block progression of malignant cells (Vaccariello et al., 1999), while UVB appears to selectively induce apoptosis in normal KC, allowing the malignant population to expand (Mudgil et al., 2003). UVB exposure of KC, human skin, or human skin equivalents reconstructed *in vitro* induces DNA damage and apoptotic KC or “sunburn” cells (SBC; (Bernerd and Asselineau, 1997; Laethem et al., 2005); reviewed in (Sheehan and Young, 2002), which ensures elimination of UV-damaged cells that potentially could become tumorigenic. Conversely, apoptosis of initiated cells would lead to tumor regression, as occurs in the majority of actinic keratoses (Rehman et al., 1996). Since dysregulation of UVB-induced apoptotic pathways and disruption of the balance between survival and apoptogenic factors in the mitochondrial death pathway leads to development of skin malignancies, a better understanding of this UVB response in initiated immortalized cells is crucial to the development of effective therapeutic strategies to control skin photocarcinogenesis.

Two main signaling pathways have been proposed to contribute to UV-induced apoptosis in KC, an extrinsic death receptor (DR)-mediated pathway and an intrinsic mitochondrial pathway; the two are not necessarily mutually exclusive and cross-talk may occur between them as a mechanism of amplification. The first signaling pathway is initiated by clustering and activation of the membrane DR, formation of a death-inducing signaling complex containing the adaptor protein FADD, which recruits and leads to autocatalytic activation of initiator procaspase-8; caspase-8 then cleaves and activates effector caspases-3 and -7.

The potential role for DR, including Fas, TNFR1, and TRAIL receptors, as well as DR ligand mediation of UVB apoptosis in KC has been reviewed (Zhuang et al., 2000). UVB may induce clustering of TNF-R1, sensitizing KC to DR-mediated apoptosis (Wehrli et al., 2000). A potential role for TNFα was demonstrated in intact animals; subcutaneous injection of a TNFα-neutralizing antibody after UVB exposure reduced numbers of SBC significantly (but not completely; (Schwarz et al., 1995). Using knockout animals, TNFR1 was shown to play a role in mediating the apoptotic response of mouse KC to UVB-induced apoptosis; the importance of the ligand-DR interaction was further demonstrated by showing that TNF-α neutralizing antibodies reduced the level of apoptosis of wild-type KC to that of the TNFR1 knockout cells (Zhuang et al., 1999).

Fas has also been suggested to play a role in mediating the apoptotic response of KC to UV. Exposure of HaCaT KC to UVA or UVB induces Fas upregulation (Banerjee et al., 2005). UV irradiation induced both Fas and FasL expression in KC, and FasL neutralizing antibody significantly reduced the apoptotic response to UV (Leverkus et al., 1997). The role of Fas-FasL interaction was also indicated using FasL

(*gld/gld*) knockout animals (Hill et al., 1999); FasL-deficient mice had significant reduction in SBC cell formation and a 14-fold higher incidence of p53 mutation accumulation in the epidermis. The importance of DR clustering and recruitment of FADD was shown to mediate UV apoptosis of HaCaT KC (Aragane et al., 1998). Since Fas-neutralizing antibodies were unable to block this effect, it was suggested that UV light directly induces clustering of DR and the activation of the apoptotic cascade. The role of UVB in sensitizing cells to TRAIL signaling has also been demonstrated (Zeise et al., 2004).

TRAIL is also expressed in intact human skin, and several studies have shown that *in vivo* UVB irradiation induces upregulation and clustering of DR (Bang et al., 2003; Bang et al., 2002). Bang *et al.* showed that UVB-irradiated normal human volunteers demonstrated cutaneous clustering of Fas within 30 min of irradiation, and recruitment of FADD; TUNEL staining was only observed in Fas-positive cells (Bang et al., 2003). Other investigators have also shown a direct upregulation of FADD itself by UV with a corresponding increase in caspase-8 cleavage and apoptosis (Kim et al., 2003). Further, adenoviral expression of antisense FADD reduced keratinocyte apoptosis. Consistent with the importance of FADD, Wu *et al.* found that overexpression of a dominant-negative mutant of FADD inhibited UV-induced apoptosis of human embryonic kidney (HEK 293) epithelial cells (Wu et al., 2002)

The mitochondrial pathway of apoptosis induced by UVB occurs primarily, although not solely, as the result of DNA damaging effects of radiation (Kulms and Schwarz, 2002). In exposed areas of human skin, UVB radiation induces cyclobutane pyrimidine dimers, 6-4 photoproducts, and cytosine photohydrates in DNA, as well as DNA strand breaks and DNA cross-links (Brash, 1997; de Gruijl et al., 2001). DNA

damage signaling to the mitochondria in some cases subsequently results in the release of cytochrome c and other apoptogenic factors (*e.g.* Smac/Diablo), which, together with Apaf-1 and ATP/dATP, triggers formation of the apoptosome and procaspase-9 activation; caspase-9 then cleaves and activates effector caspases-3 and -7, orchestrating downstream apoptotic events. The "commitment" to the release of pro-apoptotic factors from the mitochondria depends primarily on the balance between pro and anti-apoptotic members of the Bcl2 family of proteins; Bcl2 and BclxL stabilize mitochondrial integrity, while Bax and Bak destabilize this organelle. Bax has been shown to be induced, and inserts into the mitochondrial outer membrane following UVB irradiation (Miyashita and Reed, 1995). Conversely, Bcl2 overexpression in HaCaT KC blocks the effects of UVB-induced Bax activation, cytochrome c release and apoptosis, suggesting that the DR pathway does not play a dominant role in this response in this system (Assefa et al., 2003). UVB-induced DNA damage can upregulate Bax via p53-dependent or p53-independent pathways. For example, UVB induces ATR-mediated phosphorylation and stabilization of p53, which in turn can induce Bax (Shiloh, 2003), while UVB can also induce p38MAPK Bax activation in the absence of p53 (Van Laethem et al., 2004). Thus both normal and p53-mutated initiated cells can be induced to undergo apoptosis, and the latter may be one mechanism for the elimination of mutated cells (Van Laethem et al., 2004).

We have shown that UVB activates caspase-8 prior to caspase-9 in primary human KC, while in KC immortalized with HPV E6/7 caspase-9 is much more rapidly activated as a result of Bcl2 down-regulation, and proposed that resistance to apoptosis occurs at a subsequent stage (Simbulan-Rosenthal et al., 2002), reminiscent of conversion of regression-prone actinic keratoses to SCC by the upregulation of survivin

(Park et al., 2004). There is abundant experimental support for the idea that tumors evade apoptosis by acquiring inactivating mutations in proapoptotic genes (Evan and Vousden, 2001), or expressing inhibitor of apoptosis (IAP) proteins such as survivin (Altieri, 2003). The endogenous constitutive activation of Akt found in some cell lines derived from SCC has been shown to block UVB-induced apoptosis, indicating that a deregulated Akt pathway also can play a role in tumor progression during photocarcinogenesis (Decraene et al., 2004).

In the present study, we further delineated the molecular events that follow UVB irradiation in immortalized KC. To clarify whether both apical caspases-8 and-9 were necessary for UVB-induced apoptosis we pursued a specific dominant-negative approach. Retroviral constructs were utilized expressing a dominant-negative FADD mutant (FADD-DN) to block the Fas/TNF/TRAIL DR pathway or a dominant-negative caspase-9 (caspase-9-DN). Stable expression of FADD-DN in immortalized KC failed to inhibit UVB-induced activation of caspases-9, -3, and –8, and downstream apoptotic events, while inhibition of caspase-8 with zIETD-fmk blocked apoptosis. Caspase-9-DN and zLEHD-fmk both significantly suppressed caspase-9, -3 and -8 activity after UVB exposure, as well as proteolytic processing of procaspase-3 into its active form. Further, UVB-induced internucleosomal DNA fragmentation and caspase-3-mediated DFF45 cleavage was observed in UVB-treated control LHCX KC, but not in caspase-9-DN cells.

The results presented in this study identify a critical role for caspase-9 activation in the cellular response of immortalized KC to UVB-induced DNA damage. The execution of UVB-induced apoptosis requires a mitochondrial-driven pathway that depends on caspase-9 activation upstream of caspase-3 and –8, and that UVB-induced apoptosis in

HPV-immortalized human KC is DR-independent but requires both caspase-9 upstream and caspase-8 downstream, perhaps as part of an amplification mechanism.

3.2 Materials and Methods

*Cells***.** Primary KC were derived from neonatal human foreskins and grown in KSF medium supplemented with human recombinant EGF and bovine pituitary extract (Life Technologies Inc., Rockville, MD). The primary cells were infected with an amphotropic LXSN retrovirus expressing the HPV-16 open reading frame of E6 plus E7 genes. Retrovirus-infected cells were selected in G418 (100 μg/ml) for 10 days, and G418-resistant colonies were pooled from each transduction and passaged every 3 to 4 days. Primary and HPV16 E6/7-immortalized p30 human KC were grown under identical conditions to 70-80% confluency, and passaged 1:4 at equal cell densities.

*Plasmids and recombinant viruses; infection of KC with retroviral constructs***.** Replication-deficient recombinant retrovirus constructs were cloned and utilized in this study. These retrovirus constructs expressing constitutively active dominant negative FADD mutant lacking the N-terminal death effector domain (FADD-DN) and dominant negative caspase-9 (caspase-9-DN) were constructed by subcloning the cDNA for these genes into the retroviral vector pLHCX. These retroviral constructs, as well as empty vector pLHCX were then packaged and propagated in the amphotropic producer cell line ϕNX by transfection with 25 μg of the retroviral constructs using Lipofectamine 2000 (Life Tech); viral supernatants were derived, filtered, and used to infect immortalized KC in 100 mm plates in the presence of polybrene (10 μg/ml) for 4 h. Retrovirus-infected cells were selected in hygromycin (10 μg/ml) for 10 days, and

hygromycin-resistant colonies were pooled from each transduction and passaged every 4 days. Immunoblot analysis was performed on expanded pooled cells to confirm FADD-DN and caspase-9-DN expression.

*UVB Irradiation***.** KC were grown under identical conditions to 70-80% confluency, and replated at equal cell densities before UVB exposure. Cells were allowed to recover and were irradiated with indicated doses with ultraviolet light, using a UVB source with a peak wavelength of 312 nm (FS40 sunlamp (Philips) with a Kodacel cutoff filter (Kodak, France) to eliminate UV wavelengths shorter than 290 nm. Irradiation intensity was monitored by IL1400A radiometer/photometer. The doses used were selected to be within the physiological range of UV exposure of human skin, representing about three times the minimal erythema dose equivalent to moderate sunburn. In selected experiments, cells were pretreated with peptide inhibitors for caspase-3, -8, or -9 (z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk, respectively; BD Biosciences) 30 min prior to exposure to UVB. Peptides are *O*-methylated at the P1 position of aspartic acid for enhanced stability and increased cell permeability. At indicated time points after UVB irradiation, cells were harvested for further analyses.

*Fluorometric assay of caspase activity***.** Cytosolic extracts were derived from pooled floating and attached cells and subjected to fluorometric caspase-3 activity assays using fluorescent tetrapeptide substrate specific for caspases-3 (Ac-DEVD-aminomethylcoumarin (AMC), BioMol) as previously described (Simbulan-Rosenthal et al., 2002). For the fluorometric caspase-8 and -9 activity assays, the tetrapeptide substrates specific for caspases-8 and -9 (Ac-IETD-AMC and Ac-LEHD-AMC, respectively; BioMol), were utilized in essentially the same reaction assay conditions as

for caspase 3. Free AMC, generated as a result of cleavage of the aspartate-AMC bond, was monitored over 30 min with a Wallac Victor^{3}V fluorometer (Perkin Elmer) at excitation and emission wavelengths of 360 and 460 nm, respectively. The emission from each sample was plotted against time, and linear regression analysis of the initial velocity (slope) for each curve yielded the activity.

Immunoblot analysis. SDS-PAGE and transfer of separated proteins to nitrocellulose membranes were performed according to standard procedures. Membranes were stained with Ponceau S (0.1%) to verify equal loading and transfer of proteins. They were then incubated with antibodies to procaspase-3 (1:1000; BioMol) and the p17 subunit of caspase-3 (1:100; Cell Signaling), to DFF45 (1:500; BD PharMingen), to caspase-9 (1:1000; Calbiochem), or to FADD (1:500; Calbiochem). Immune complexes were detected by subsequent incubation with appropriate horseradish peroxidase– conjugated antibodies to mouse or rabbit IgG (1:3000) and enhanced chemiluminescence (Pierce, Rockford, IL).

Analysis of DNA fragmentation. Cells were harvested and lysed in 0.5 ml of 7 M guanidine hydrochloride. The lysate was mixed with 1 ml of Wizard Miniprep resin (Promega, Madison, WI), incubated at room temperature for 15 min with occasional mixing and then centrifuged. The resulting pellet was resuspended in 2 ml of washing solution (90 mM NaCl, 9 mM Tris-HCl (pH 7.4), 2.25 mM EDTA, 55% (v/v) ethanol), drawn by vacuum through a Wizard Minicolumn (Promega) mounted onto a vacuum manifold, washed twice with 4 ml of washing solution and dried by centrifugation. DNA was eluted from the column with deionized H_2O and residual RNA was removed in the eluate by incubation with 10 µg of RNase A at 37ºC for 30 min. DNA samples were

then loaded onto a 1.5% agarose gel in TBE buffer and subjected to electrophoresis at 4 V/cm. DNA ladders were visualized by staining with ethidium bromide (0.5 µg/ml) and images were captured with the Kodak EDAS 120 (Kodak) gel documentation system.

Statistical Analysis. For caspase activity assays, data derived from 3 experiments were compared using 2-way ANOVA tests with Bonferroni post-tests for significance. P values of <0.05 were considered statistically significant. The results are representative of at least 3 independent experiments with reproducible results.

3.3 Results and Discussion

Normal keratinocytes have been shown to block progression of malignant cells in organotypic models (Vaccariello et al., 1999), while UVB appears to selectively induce apoptosis in normal KC, allowing the malignant population to expand (Mudgil et al., 2003). Thus, apoptosis of normal KC may contribute to tumor progression, while apoptosis of initiated/immortalized cells could result in tumor regression. The pathways involved in each of these processes are of obvious importance to the understanding of carcinogenesis. However, different studies have yielded seemingly conflicting results concerning the relative contributions of the DR *vs*. the mitochondrial pathway of apoptosis. It is certainly conceivable that cell type determines the mode of cell death. Based upon our previous studies suggesting that upon HPV-immortalization, caspase-9 is activated earlier and at lower doses of UVB, we have now tested the individual contributions of FADD and caspase-9 using dominant-negative retroviral expression vectors as well as with peptide inhibitors. Our results indicate that caspase-9-DN

suppresses UVB apoptosis in KC immortalized with HPV16 E6/7, and that the DR plays no role in the process in these cells. Our results using physiologically relevant HPV-immortalized cells (see below) are in agreement with those of Sitailo *et al.*, who found that caspase-9-DN blocked UVB-induced apoptosis in spontaneously immortalized HaCaT cells, as well as in primary human KC (Sitailo et al., 2002). Additionally, since other studies have also implicated Fas/TNF/TRAIL DR in this process we have also tested the individual contribution of DR to the apoptotic response using FADD-DN. To our knowledge, this study is the first to use both of these highly specific dominant-negatives and peptide inhibitors in one cell type to test each of these pathways side-by-side in response to UVB. Furthermore, we show that while caspase-8 activation is an event that occurs only downstream of the mitochondrial pathway in immortalized cells (since caspase-9-DN, but not FADD-DN is able to block the activation of caspase-8), caspase-8 is nonetheless required for the maximal apoptotic response. Other studies have also shown that caspase-8 can be activated downstream of caspase-9 via both caspase-3-dependent (Slee et al., 1999) and caspase-3-independent pathways (Pirnia et al., 2002). DR-independent FADD has also been implicated in caspase-8 activation downstream of mitochondrial events during anoikis (Rytomaa et al., 1999), or following treatment with anticancer drugs (Micheau et al., 1999; Wesselborg et al., 1999). Since FADD-DN did not block UVB-induced caspase-8 activation in our system, downstream cleavage via a caspase-3-dependent pathway rather than induced proximity (FADD-dependent) may be involved in the activation of caspase-8 by UVB. Importantly, when caspase-8 activity is blocked by IETD-fmk apoptosis is attenuated, demonstrating a role for caspase-8 in amplifying UVB-induced apoptosis of HPV-immortalized KC, possibly *via* cleavage and activation of Bid.

While solar simulated light was not utilized in the present study, a filter that mimics the earth's atmosphere by eliminating UVC was employed. Additionally, a UVB irradiance of 480 J/m^2 which is equivalent to approximately twice the minimal erythema dose (about one-half hour summertime exposure in Baltimore, MD (Heisler et al., 2004). Nonetheless, UVA has been shown to contribute to both KC apoptosis and photocarcinogenesis (Larsson et al., 2005). Thus, additional studies utilizing both UVA and UVB, as well as human skin grafted onto nude mice are currently underway to determine the relative role of DR and mitochondrial pathways in apoptosis and carcinogenesis of primary and immortalized KC.

Immortalization is thought to be an early step in this process that involves the selection of a population of cells that express hTERT and progress to the next stage of cancer; thus preneoplastic (AK and porokeratosis) as well as neoplastic nonmelanoma (SCC and BCC) skin lesions express hTERT (Park et al., 2004). We chose to use HPV16 immortalization because of its physiological relevance as well as its utility as a defined agent for immortalization. A number of different HPV types are found in high percentage of BCC and SCC in immunosuppressed individuals (Shamanin et al., 1996). Similarly, a range of HPV types have been isolated from actinic keratoses and SCC from epidermodysplasia verruciformis (EV) patients (Bouwes Bavinck et al., 2000). Further, HPV may likely play a role in these cutaneous carcinomas from immunocompetent non-EV patients as well (Shamanin et al., 1996; zur Hausen, 1996). While long implicated in the etiology of anogenital cancer, HPV16 and 18 are now associated with up to 25% of non-genital Bowen's Disease of the skull, foot, and periungual regions of the finger (Guerin-Reverchon et al., 1990; Kettler et al., 1990); the latter lesion represents a significant fraction of all skin cancer cases (Eliezri et al., 1990). As sensitive, strain-

specific detection methods become more commonly utilized HPV-16 is being found in other skin cancers as well, including SCC of the lip (Kawashima et al., 1990). In most cases of these nongenital HPV skin cancers, carcinomas occur in sun-exposed sites, indicating cooperation between UV and HPV, including HPV16. Since the HPV16 E6 protein participates in the degradation of p53 (Thomas et al., 1999), E6 may in part play the same role as UV-induced p53 mutations during the initiation phase of skin carcinogenesis (Sedman et al., 1992). Interestingly, while E6 derived from HPVs that are more traditionally classified as cutaneous, including HPV77, do not degrade p53, they do interfere with its ability to upregulate the expression of certain key proapoptotic genes in response to UVB, including Fas, PUMAb, Apaf-1, and PIG3 (Giampieri et al., 2004). In most epidermal malignancies, the p16-Rb pathway is also inactivated; in our system Rb is inactivated by E7. In addition to UVB p53-independent activation of p38 MAPK and Bax (Van Laethem et al., 2004), sequestering of Rb by E7 would allow E2F1 to upregulate p73 (Irwin et al., 2000; Stiewe and Putzer, 2000), Apaf1 (Furukawa et al., 2002), as well as a number of BH3-only family members, including PUMA, Noxa, Hrk/DPS, and Bim (Hershko and Ginsberg, 2004), which, coupled with lower Bcl2 levels in HPV 16 E6/7 immortalized KC would lead to activation of caspase-9. E2F1 also induces other less well-studied inducers of apoptosis via p53-independent pathways including galectin, and actinin a (which activates the apoptotic DNAse Y; (Li et al., 2005).

Previous studies comparing the response of primary KC to their immortalized counterparts have utilized cells that are tumor-derived or that have been cultured for long periods of time, such as spontaneously immortalized HaCaT cells. The use of immortalized cell lines such as HaCaT involve a number of undefined changes that occur over time in culture. We also found that NcoI cells, KC immortalized with HPV16

E6/7 but cultured for an undetermined period of time in the presence of intermediate calcium (0.5 mM *vs*. 0.1 mM), were completely resistant to UVB-induced apoptosis (data not shown). NcoI cells may therefore represent a later irreversible stage of tumorigenesis coinciding with, among other things a loss of the apoptotic response, and that the earliest stages, *i.e.* immortalization, correspond with decreased cell cycle control and DNA repair, and concomitant selection for a population with decreased genomic stability. To avoid cell culture artifacts that may arise from the use of well-established lines such as HaCaT cells, cells were immortalized with a defined and physiologically relevant agent, HPV16 E6/7 (above), and utilized at passage 30 (60 population doublings), a minimum passage to be considered immortal (Kiyono et al., 1998; Rheinwald and Green, 1975). In addition, immortalized cells used in these studies were derived from pooled clones since KC were transduced with a high-titer retroviral vector expressing HPV16 E6/7.

The idea that the anti-apoptotic phenotype arises or continues to evolve after immortalization is supported by *in vivo* studies in which it was found that p53, p63 (a p53 homologue expressed in replicating KC), and hTERT are expressed in the preneoplastic lesions AK and porokeratosis, as well as in SCC *in situ* and invasive SCC. However, upregulation of the IAP survivin, which is abundantly expressed in most solid and hematologic malignancies, is only found in SCC but not in AK or porokeratosis (Park et al., 2004). Similarly, Akt is constitutively activated in some SCC, and blocks UVB-induced apoptosis (Decraene et al., 2004). Akt activation phosphorylates the proapoptotic protein Bad, sequestering it in the cytosol with 14-3-3 proteins, and consequently preventing cytochrome c release and activation of caspases-9 and -3 (Wang et al., 2003). The importance of these anti-apoptotic proteins involved primarily

in the mitochondrial pathway in cutaneous carcinogenesis has been verified in animal models. KC of transgenic mice overexpressing survival proteins Bcl2 or BclxL exhibit a UVB-resistant phenotype as well as a predisposition to skin cancer (Nickoloff et al., 2002). Survivin, which is undetectable in adult skin but upregulated in malignant KC, when expressed transgenically in skin leads to resistance to UVB-induced cell death by suppression of mitochondrial apoptosis (Grossman et al., 2001). Conversely, UVB-sensitivity to apoptosis is increased by alteration of NFkappaB signaling in KC or transgenic expression of a super suppressor IkappaB in skin (Nickoloff et al., 2002). Akt activates the NFkappaB pathway, and is itself a downstream target of NFkappaB (Madrid et al., 2001; Meng et al., 2002).

We postulate that in addition to p53-dependent apoptosis, KC have a built in fail-safe (almost) mechanism whereby immortalizing agents render cells extremely sensitive to apoptosis and only further stable genetic or epigenetic events (*e.g.* upregulation of survivin or constitutive Akt activation) allow the progression of a small subset of immortalized cells, whereas the majority undergo p53-independent apoptosis and regression.

4. Experiment 1: Continuation of Studies utilizing peptide caspase inhibitors to block SM-induced apoptosis and vesication in grafted human skin

4.1 Introduction

We have successfully established that caspases play a role in SM toxicity in cultured cells throughout the Phase I and Phase II periods of the research. In the current Phase II research, we have utilized specific and potent inhibitors of different caspases to determine the most effective means of preventing toxicity in vitro and vesication in vivo. Thus, we obtained tetrapeptide inhibitors to each of the caspases and treated keratinocytes for 30 min prior to SM exposure. In addition to determining total SM toxicity, we were also able to delineate the specific pathway for the activation of each of the caspases. In the previous annual report, we showed that when primary keratinocytes were treated for each of the caspase inhibitors, prior to SM exposure and assayed for activation of each caspase, IETD and LEHD (caspase-8 and caspase-9 inhibitors) were found to be the most effective caspase inhibitors for human keratinocytes in culture. Surprisingly, these worked more effectively than the pan-caspase inhibitor, ZVAD. Using these peptide caspase inhibitors, we were also able to delineate that caspase-8 activation is most upstream, and activates caspase-9 via "crosstalk" between the death receptor and mitochondrial pathways, dependent on activation of caspase-3 (via Bid cleavage**). In the current annual report for 2006, we have now utilized these inhibitors as compounds for testing in the *in vivo* human skin graft for their ability to block SM vesication.** We have previously used primary human keratinocytes to establish a histologically and immunocytochemically normal epidermis when grafted onto nude mice (Rosenthal et al., 1995; Rosenthal et al., 1998a; Rosenthal et al., 2000). The correct expression of human keratins K1, K10 and K14

have been confirmed in the grafted epidermis utilizing human keratin-specific antibodies (Rosenthal et al., 1995). To test the effects of the peptide inhibitors of caspases on apoptosis and vesication in intact human epidermis, we utilized this system to graft normal human skin onto the back of athymic mice, and 6-8 weeks after grafting, we inhibited the activity of caspases in vivo by topical application of the inhibitors at the graft site 30 min prior to SM exposure. These human grafts were then exposed to SM by vapor cup method, and showed a vesication response.

The immediate purpose is to devise prevention or treatment strategy for soldiers and civilians who may be exposed to this debilitating substance, and the long term goal is to understand how similar, warfare and non-warfare alkylating agents (UV, chemical carcinogens), are involved in epithelial lesions and carcinogenesis. In order to do this, we are using the following model system:

Nude mice grafted with human keratinocytes. The keratinocytes can be stably transfected with control vector, or vector expressing a “dominant negative” inhibitor of the Fas receptor pathway (truncated FADD protein). The tetrapeptide inhibitors and controls will be dissolved in DMSO at a concentration of 50 μM and 75 μl will be applied with a micropipettor to 6-7 week grafted skin on the morning prior to shipment to Aberdeen. The peptide inhibitors include:

a. *Z-DEVD-FMK (Benzyloxycarbonyl-aspartyl-glutamyl-valyl-aspartyl-fluoromethyl ketone) is a cell and skin-permeable non-pain-inducing specific inhibitor of caspase-3, the executioner caspase of apoptosis. In cultured cells, this inhibitor partially blocks the toxicity of SM to keratinocytes and is the converging point for most apoptotic pathways.

b. *Z-IETD-FMK (Benzyloxycarbonyl-isoleucyl-glutamyl-threonyl-aspartyl-fluoromethyl ketone) is a cell and skin-permeable non-pain-inducing specific inhibitor of caspase-8, the initiator caspase of the death receptor pathway. In cultured cells, this inhibitor blocks the toxicity of SM to keratinocytes and also blocks the activation of caspase-3.

c. *Z-LEHD-FMK (Benzyloxycarbonyl-leucyl-glutamatyl-histidinyl-aspartly-fluoromethyl ketone) is a cell and skin-permeable non-pain-inducing specific inhibitor of caspase-9, the initiator caspase of the mitochondrial pathway. In cultured cells, this inhibitor partially blocks the toxicity of SM to keratinocytes, and in the presence of FADD-DN may hopefully inhibit SM toxicity *in vivo*.

d. *Z-VAD-FMK (Benzyloxycarbonyl--valyl-alanyl-aspartyl-fluoromethyl ketone) is a cell and skin-permeable non-pain-inducing specific inhibitor of all caspases. Surprisingly, in cultured cells, this inhibitor does not block the toxicity of SM to keratinocytes as effectively as the above inhibitors.

e. *Z-FA-FMK (Benzyloxycarbonyl--phenylalanyl-asparty-fluoromethyl ketone) is a cell and skin-permeable non-pain-inducing dipeptide that does not inhibit caspases but resembles the above inhibitors and thus serves as a good negative control. As expected, in cultured cells, this inhibitor does not block the toxicity of SM to keratinocytes.

f. DMSO- vehicle control.

SM exposure: After the graft is complete (6-7 weeks), the animals are shipped, under the supervision of DCM and the PI, to USAMRICD at the Aberdeen Proving Ground. The goal of project is to determine the role of Fas in SM blistering. There is no cell culture model that can even approximate the blistering response, including

monolayer, or stratified raft cultures of keratinocytes. In order to study blistering, one needs intact, physiological normal epidermis, and intact basal lamina (since this is the primary site of detachment of the skin), and an underlying dermis that contributes attachment proteins. In addition, it is important to know if an immune response and lymphocytic infiltration into the skin occurs following different treatments; this can only be studied in the intact animal.

We have data that show that we can graft genetically modified keratinocytes (i.e., containing plasmids that alter the function of Fas as described above) and these keratinocytes form a well-organized stratified squamous epithelium that is indistinguishable from normal human skin both histologically and biochemically. The grafted skin also forms a bona fide basal lamina above the dermis, and is thus a good model to study the blistering effects of SM.

The mice represent 6 peptide inhibitor treatment groups (Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK, Z-VAD-FMK, Z-FA-FMK, and no inhibitor) x 15 replicate mice per treatment group (10 mice with 6 min. vapor cup exposure plus 5 untreated controls) = 90 nude mice total. A sample size of 5 in the control group and 10 in the treated group will have 88% power to detect a shift in histopathology scores of 1 category using a Wilcoxon (Mann-Whitney) rank-sum test with a 0.050 two-sided significance level. In addition to histological analysis for blistering, inflammation, and necrosis, all SM-exposed and control skin grafts are analyzed by histological and immunocytochemical analysis. All grafts were utilized.

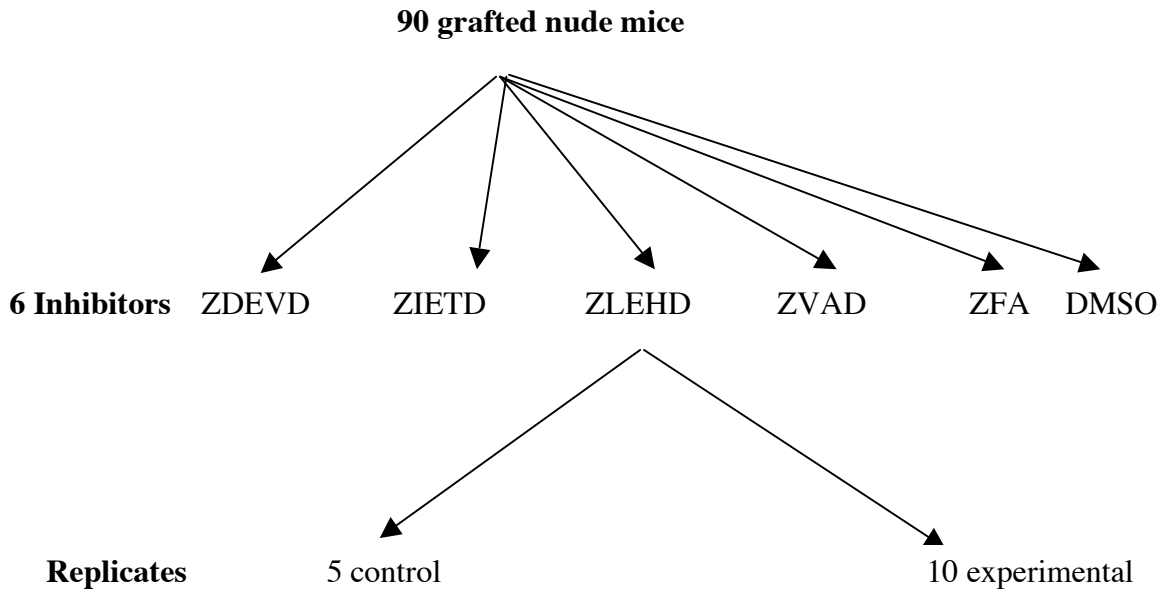

FIG. 1 Strategy for determining effective caspase inhibitors

Abbrev.	Inhibitor	Specificity	Caspase "Type"
DEVD	ZDEVD-FMK	Caspase-3	Executioner
IETD	ZIETD-FMK	Caspase-8	Upstream Death Receptor
LEHD	ZLEHD-FMK	Caspase-9	Upstream Mitochondrial
ZVAD	ZVAD-FMK	All	All Caspases
ZFA	ZFA-FMK	None	Control Compound for all above
DMSO	Untreated		

4.2 Materials and Methods

a. Culture of primary human keratinocytes. Culture of primary human keratinocytes cells were performed as described in previous sections.

b. Chemicals. SM (bis-(2-chloroethyl) sulfide; >98% purity) was obtained from the US Army Edgewood Research, Development and Engineering Center.

c. Grafting Protocols and Exposure of Human Skin Grafts to SM. A piece of skin (1 cm diameter) was removed from the dorsal surface of athymic mice, and a cell pellet containing 8 x 10^6 fibroblasts + 5 x 10^6 keratinocytes (KC) was pipetted on top of the muscular layer within a silicon dome to protect the cells. The dome was removed after a week and the graft was allowed to develop for 6-8 weeks. SM exposure was performed by placing a small amount of SM liquid into an absorbent filter at the bottom of a vapor cup, which was then inverted onto the dorsal surface of the animal, to expose the graft site to the SM vapor. Frozen and fixed sections were derived from punch biopsies taken from the graft site. Histological analysis of the SM-exposed human skin grafts transplanted onto nude mice was also performed utilizing an end point of micro or macro blisters or SM-induced microvesication.

d. Assays for In Vivo Markers of Apoptosis on Human Skin Grafts. Paraffin-embedded sections derived from SM-exposed human skin grafts were subjected to analysis for markers of *in vivo* apoptosis, including immunofluorescence microscopy with antibodies to the active form of caspase-3 (Cell Signaling). Sections were deparaffinized, incubated overnight in a humid chamber with antibodies to active caspase-3 (1:250) in PBS containing 12% BSA, washed with PBS, incubated for 1 h with biotinylated anti-mouse IgG (1:400), washed again, incubated for 30 min with streptavidin-conjugated Texas red (1:800 dilution), mounted with PBS with 80% glycerol, and observed with a Zeiss fluorescence microscope. DNA breaks late stage of apoptosis were detected *in situ* using a Klenow fragment-based assay system (DermaTACS; Trevigen). For fixation, slides were dried for 2 h on a slide warmer at 45° C, rehydrated in 100%, 95%, then 70% ethanol, washed in PBS, fixed in 3.7% buffered

formaldehyde for 10 min at room temp, and washed in PBS. Slides were then incubated with 50 µl of Cytonin for 15 min, washed in water, immersed in quenching solution containing 90% methanol and 3% H_2O_2 for 5 min, washed with PBS, incubated in TdT labeling buffer, and visualized under a bright field microscope.

4.3 Results and Discussion

Human skin grafts transplanted onto nude mice have been used successfully to examine SM-induced biochemical alterations, utilizing an end point of micro or macro blisters (Gross et al., 1988; Meier et al., 1984; Papirmeister et al., 1991; Petrali et al., 1990; Smith et al., 1990; Smith et al., 1991; Smulson, 1990; van Genderen and Wolthuis, 1986). The model has also been used to assess the protection afforded against SM-induced biochemical alterations by the systemically administered inhibitors. Frozen and fixed sections derived from graft sites of these animals were thus analyzed for the apoptosis markers.

We now demonstrate that upon histological analysis of SM-exposed animals grafted with primary KC, SM microvesication can be reduced by topical application of the pan-caspase peptide inhibitor zVAD-fmk. While there was no difference in the DMSO (vehicle)-treated and ZFA-treated control skin grafts, there was a notable decrease in the amount of microvesication in grafts treated with zVAD-fmk (**Figs. 2-4**). A summary of the pathology report is shown in Table 1.

Table 1

		Pustular Epidermitis	Epidermal Necrosis	Vesicle	Follicular damage	Dermal Necrosis	Punch Weight
Graft	DMSO	1	3.5	2.5		3.5	79.8
	ZFA	0	3.7	1.7		4	74.8
	ZVAD	0.7	2.3	0.7		3.7	79.2
Recipient	DMSO	2.5	3.5	2.5	2.5	3	
	None	1.5	4	0.5	3.5	4	
	ZFA	1.3	3.7	0.7	3.3	3.7	
	ZVAD	1.7	3.7	1	3.3	3.7	

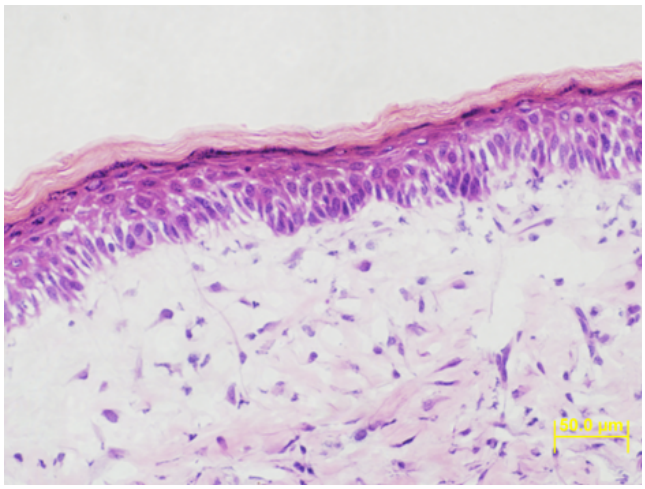

FIG. 2: Skin Graft treated with ZVAD prior to SM

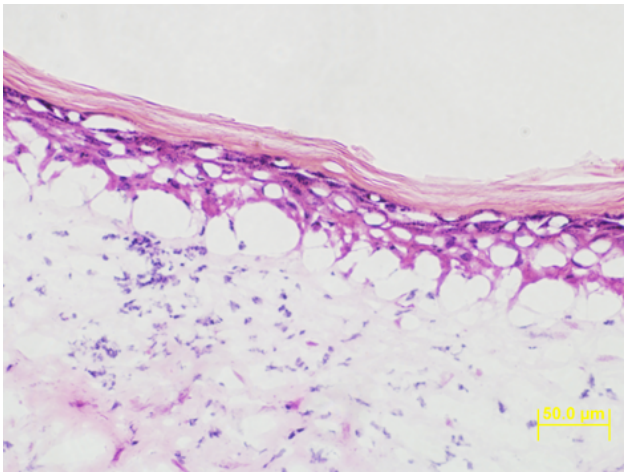

FIG. 3: Skin Graft pretreated with DMSO

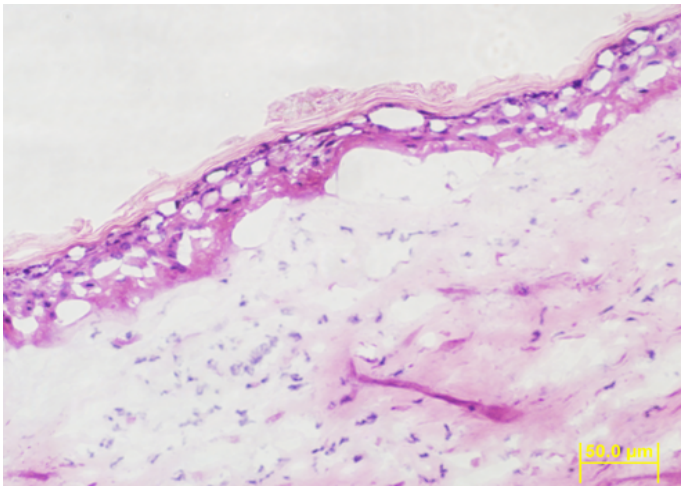

FIG 4: Skin Graft pretreated with ZFA

5. Experiments 2-5: Utilizing peptide inhibitors to caspases-3, -8, and -9 vs. zVAD to block SM-induced apoptosis in keratinocytes and vesication in grafted normal and FADD-DN human skin

5.1 Introduction

SM induces human keratinocyte (KC) apoptosis, and this response is altered by inhibiting either the mitochondrial (CaM-Bcl-2-caspase) or death receptor (Fas-FADD-caspase) pathways via expression of dominant-negative or antisense constructs. Specific potent tetrapeptide caspase inhibitors were used to determine the most effective means of preventing SM toxicity *in vitro* and vesication *in vivo*, as well as to delineate the specific pathway for the activation of each of the caspases. Primary KC were pretreated with caspase inhibitors for 30 min prior to SM exposure, and assayed for activation of caspases-3, -8, and -9, and apoptotic nuclear fragmentation by Hoechst staining. zIETDfmk and zLEHDfmk (caspase-8 and caspase-9 inhibitors) as well as the pan-caspase inhibitor, zVADfmk were found to be the most effective inhibitors, compared to the inactive control peptide zFA. Caspase-8 activation was delineated to be upstream, and activates caspase-9 via “crosstalk” between the death receptor and mitochondrial pathways, dependent on activation of caspase-3. These caspase inhibitors were then used in the *in vivo* human skin graft for their ability to block SM vesication. Primary human KC was grafted onto the back of athymic mice, and 3-4 weeks after grafting, the activity of caspases was inhibited *in vivo* by topical application of the peptide inhibitors at the graft site 30 min prior to SM exposure. These human grafts were then exposed to SM by vapor cup method, and frozen sections derived from punch biopsies of SM-exposed graft sites were subjected to

histological analysis utilizing an end point of micro or macro blisters or SM-induced microvesication. Results show that SM microvesication can be reduced by topical application of the pan-caspase peptide inhibitor zVAD-fmk. Compared to DMSO (vehicle)-treated skin grafts, there was a decrease in the amount of microvesication in grafts treated with zVADfmk. Sections derived from SM-exposed human skin grafts were further subjected to analysis for markers of *in vivo* apoptosis, including immunofluorescence microscopy with antibodies to active caspases-3, -8, and -9, and detection of apoptotic DNA breaks by *in situ* TUNEL staining. Apoptotic cells were concentrated in the areas of microvesication. SM induced apoptotic DNA breaks primarily in the basal cells of DMSO-treated control human skin grafts, but was markedly attenuated in the grafts treated with zVADfmk. Pretreatment of the skin grafts with individual caspases-8 or -9 peptide inhibitors were less effective in modulating this response. SM activates both a death receptor and mitochondrial apoptotic pathway resulting in the activation of multiple caspases and apoptosis of basal cells, contributing to the vesication response. Inhibition of one of the caspases appears to be insufficient in blocking the cytotoxic effects because other caspases are still active and compensate, thus, the pan-caspase inhibitor zVAD-fmk represents a promising therapeutic agent to reduce SM injury.

5.2 Materials and Methods

a. Culture of primary human keratinocytes. Culture of primary human keratinocytes cells were performed as described in previous sections.

b. Chemicals. SM (bis-(2-chloroethyl) sulfide; >98% purity) was obtained from the US Army Edgewood Research, Development and Engineering Center.

c. Grafting Protocols and Exposure of Human Skin Grafts to SM. A piece of skin (1 cm diameter) was removed from the dorsal surface of athymic mice, and a cell pellet containing 8 x 10^6 fibroblasts + 5 x 10^6 keratinocytes (KC) was pipetted on top of the muscular layer within a silicon dome to protect the cells. The dome was removed after a week and the graft was allowed to develop for 6-8 weeks. SM exposure was performed by placing a small amount of SM liquid into an absorbent filter at the bottom of a vapor cup, which was then inverted onto the dorsal surface of the animal, to expose the graft site to the SM vapor. Frozen and fixed sections were derived from punch biopsies taken from the graft site. Histological analysis of the SM-exposed human skin grafts transplanted onto nude mice was also performed utilizing an end point of micro or macro blisters or SM-induced microvesication.

d. Assays for In Vivo Markers of Apoptosis on Human Skin Grafts. Paraffin-embedded sections derived from SM-exposed human skin grafts were subjected to analysis for markers of *in vivo* apoptosis, including immunofluorescence microscopy with antibodies to the active form of caspase-3 (Cell Signaling). Sections were deparaffinized, incubated overnight in a humid chamber with antibodies to active caspase-3 (1:250) in PBS containing 12% BSA, washed with PBS, incubated for 1 h with biotinylated anti-mouse IgG (1:400), washed again, incubated for 30 min with streptavidin-conjugated Texas red (1:800 dilution), mounted with PBS with 80% glycerol, and observed with a Zeiss fluorescence microscope. DNA breaks late stage of apoptosis were detected *in situ* using a Klenow fragment-based assay system (DermaTACS; Trevigen). For fixation, slides were dried for 2 h on a slide warmer at 45° C, rehydrated in 100%, 95%, then 70% ethanol, washed in PBS, fixed in 3.7% buffered

formaldehyde for 10 min at room temp, and washed in PBS. Slides were then incubated with 50 µl of Cytonin for 15 min, washed in water, immersed in quenching solution containing 90% methanol and 3% H_2O_2 for 5 min, washed with PBS, incubated in TdT labeling buffer, and visualized under a bright field microscope.

5.3 Results and Discussion

Human skin grafts transplanted onto nude mice have been used successfully to examine SM-induced biochemical alterations, utilizing an end point of micro or macro blisters (Gross et al., 1988; Meier et al., 1984; Papirmeister et al., 1991; Petrali et al., 1990; Smith et al., 1990; Smith et al., 1991; Smulson, 1990; van Genderen and Wolthuis, 1986). The model has also been used to assess the protection afforded against SM-induced biochemical alterations by the systemically administered inhibitors. Frozen and fixed sections derived from graft sites of these animals were thus analyzed for the apoptosis markers.

We now demonstrate that upon histological analysis of SM-exposed animals grafted with primary KC, SM microvesication can be reduced by topical application of the pan-caspase peptide inhibitor zVAD-fmk. While there was no difference in the DMSO (vehicle)-treated and ZFA-treated control skin grafts, there was a notable decrease in the amount of microvesication in grafts treated with zVAD-fmk.

Graft	Pustular Epidermitis	Epidermal Necrosis	Vesicle
DMSO	1	3.5	2.5
zFA	0	3.7	1.7
zVAD	0.7	2.3	0.7

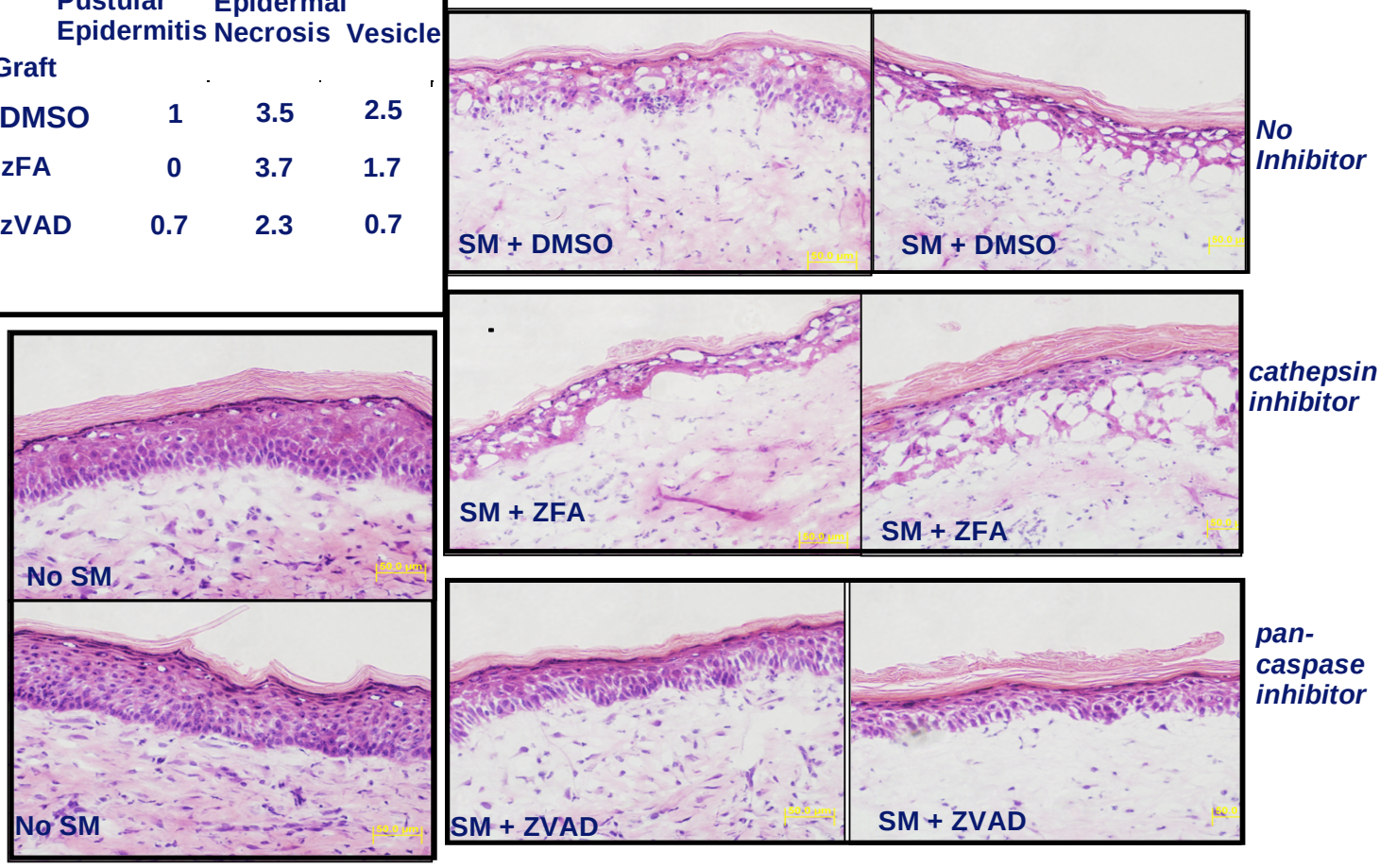

***Fig. 5 (Expt. 2):* Grafted human skin pretreated with the pan-caspase inhibitor zVAD-fmk show reduced vesication after SM exposure.** *0= no necrosis; 1=5% or less; 2=10-40%; 3=50-80%; 4=90-100%*

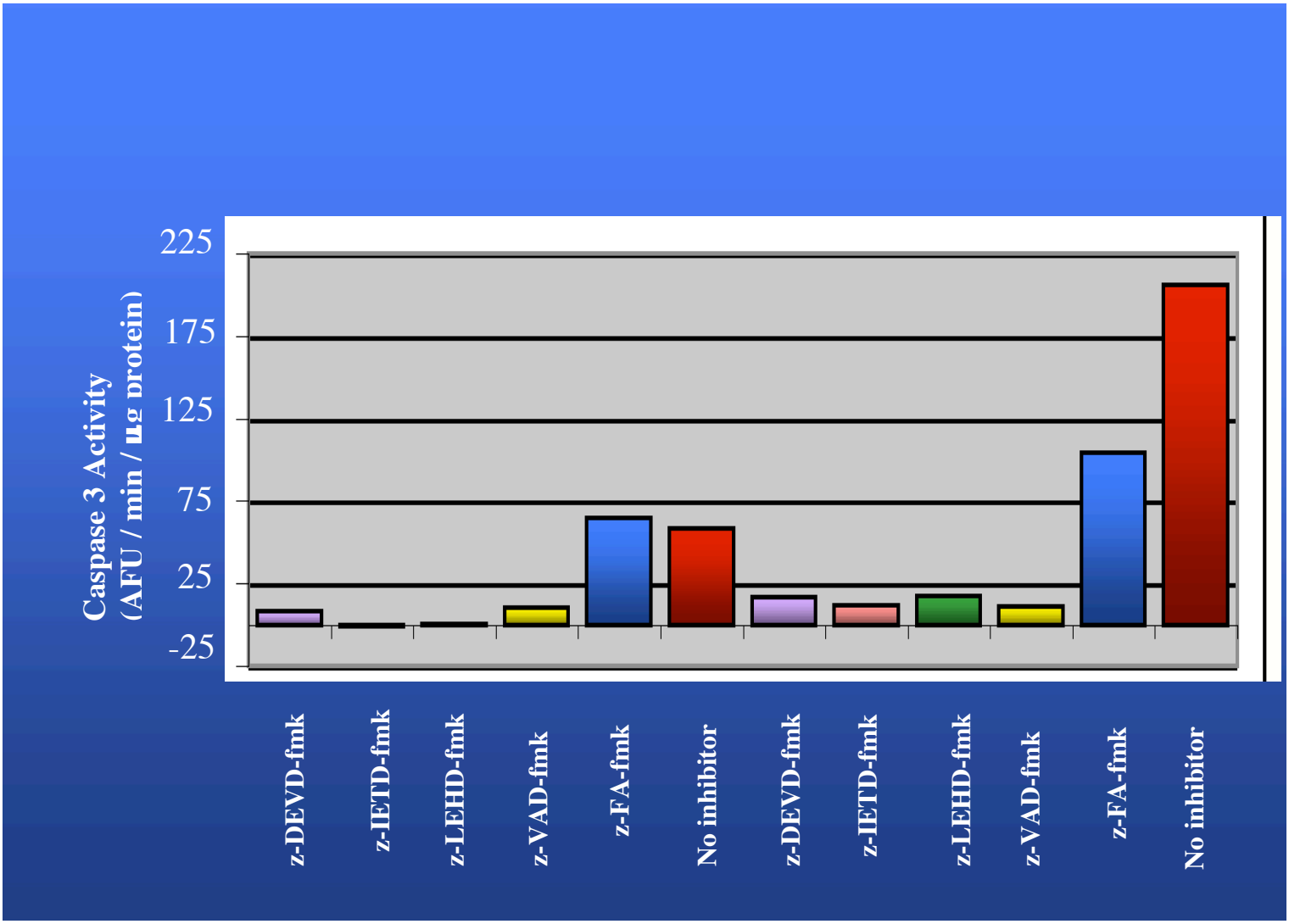

Fig. 6 (Exp. 3) Peptide inhibitors of caspase -3, -8, and -9 block caspase-3 activation in human keratinocytes exposed to SM

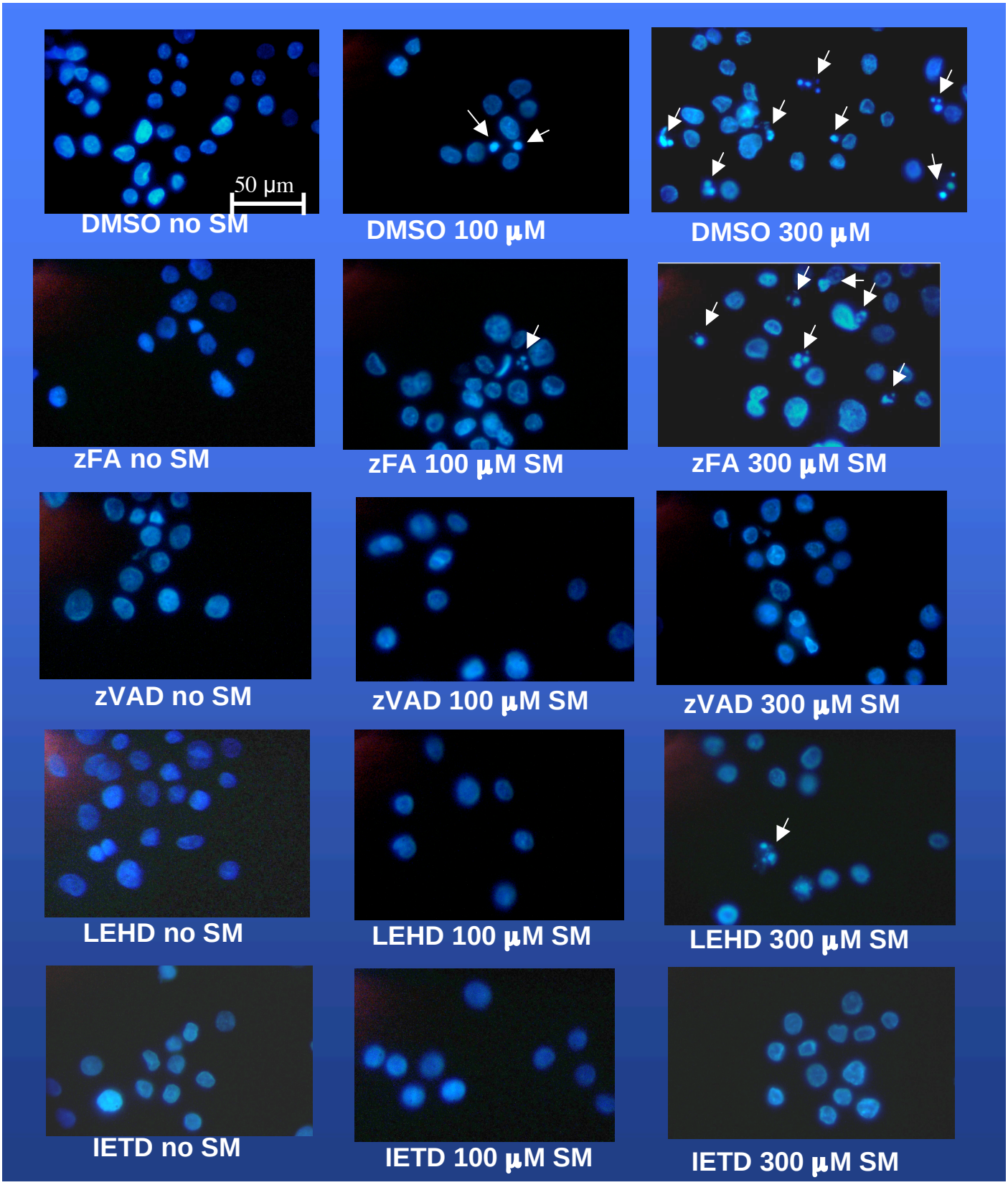

Fig. 7 (Exp. 3) Inhibitors of caspase -3, -8, -9 attenuate apoptotic nuclear fragmentation in human KC exposed to SM

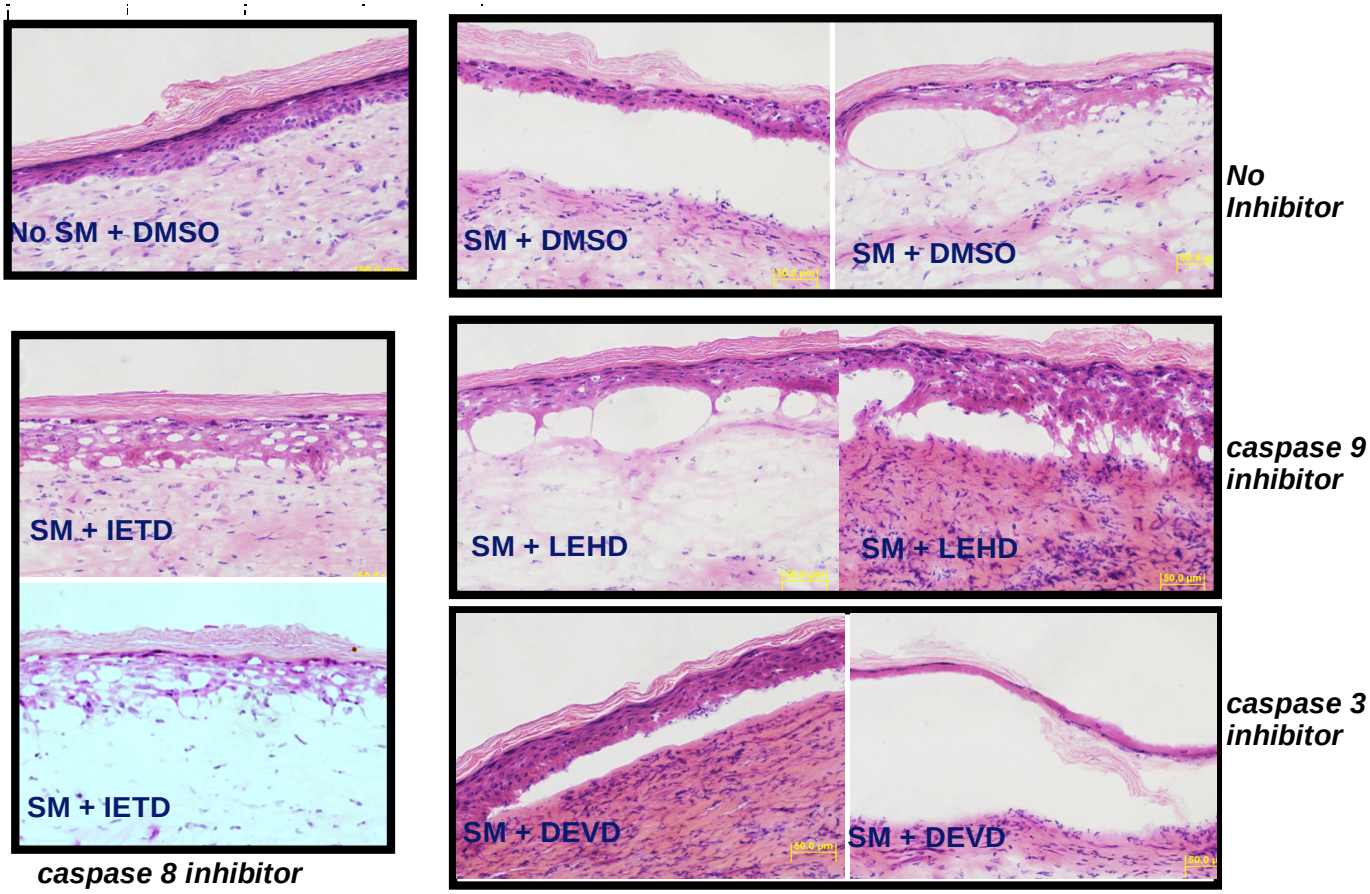

Fig. 8 (Expt. 3,4) Individual caspases-3 and -9 inhibitors do not suppress SM vesication

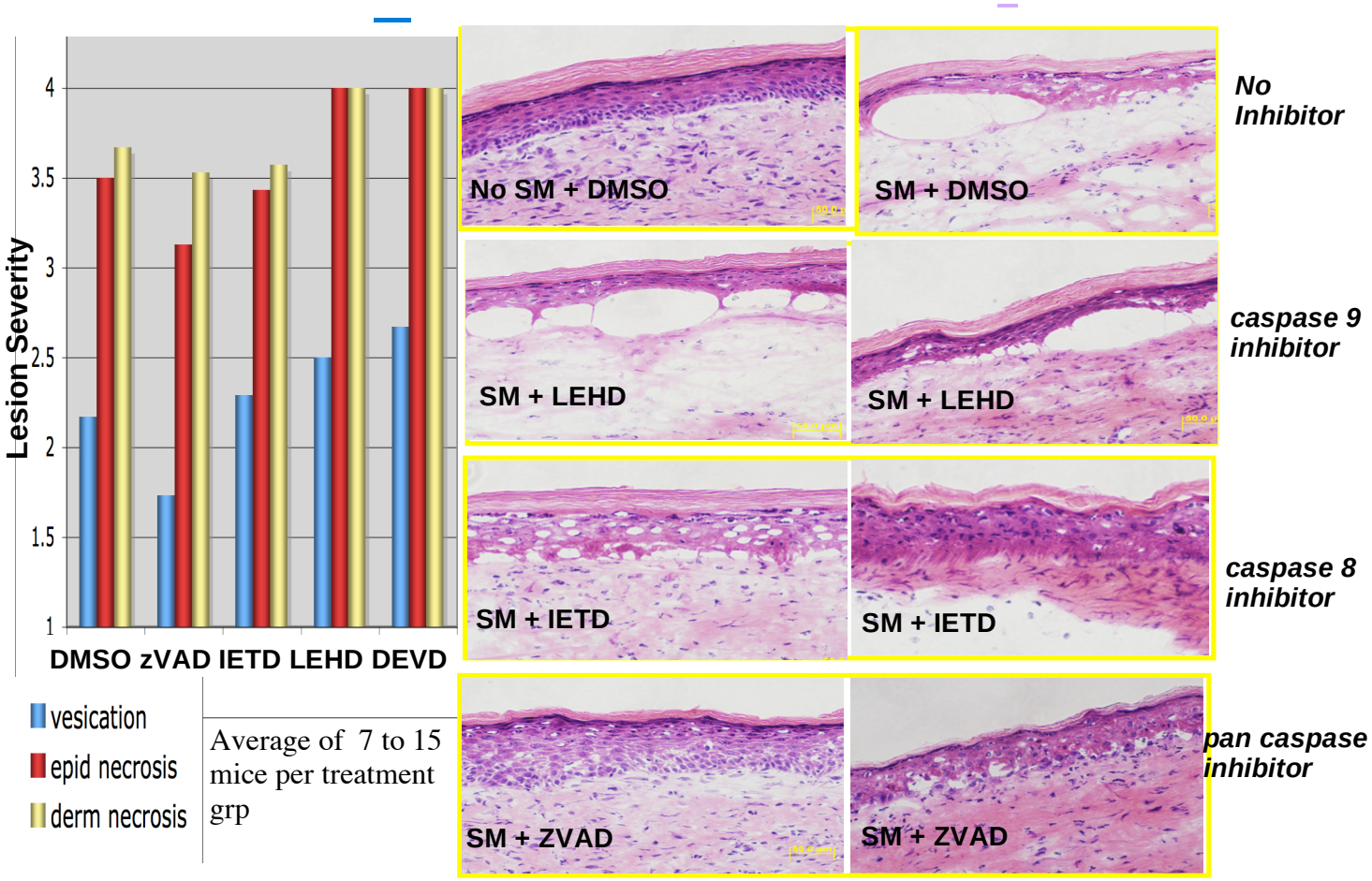

Fig. 9 (Expt. 3, 4): zVAD-fmk can decrease lesion severity

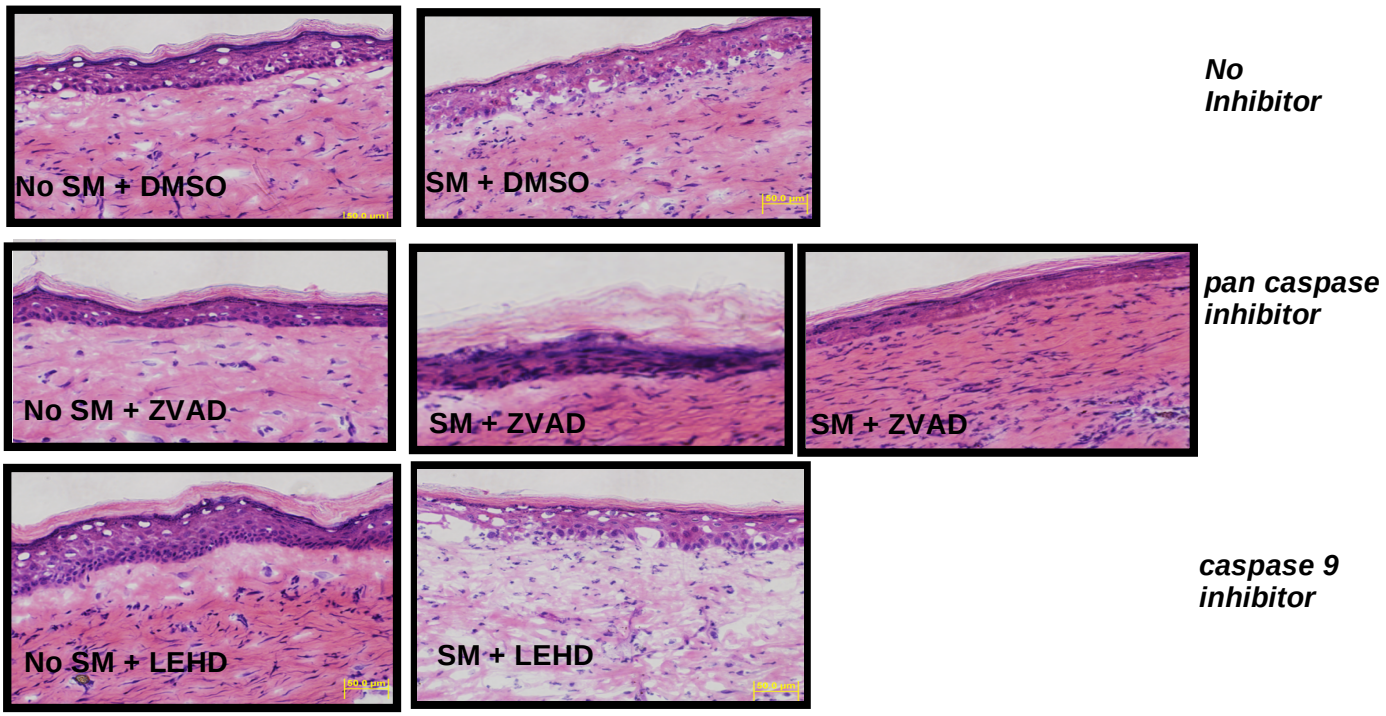

Fig.10 (Expt 5) FADD-DN expressing keratinocytes pretreated with zVAD are resistant to SM vesication

6. CONCLUSIONS

- ✓ ***Inhibitors of caspase -8 and -9 suppress SM-induced activation of caspase -8 and -9 in cultured keratinocytes***

- ✓ ***Inhibitors of caspase -3, -8, -9 suppress activation of caspase-3 and attenuate apoptotic nuclear fragmentation in human KC exposed to SM***

- ✓ ***Grafted human skin pretreated with the pan-caspase inhibitor zVAD-fmk show reduced vesication after SM exposure by vapor cup***

- ✓ ***Individual caspases-3, -8 and -9 inhibitors do not suppress SM vesication***

- ✓ ***A combination of FADD-DN and zVAD provides more effective protection against SM vesication in grafted human skin***

- ✓ ***Inhibition of one of the caspases is insufficient in blocking the cytotoxic effects because other caspases are still active and compensate, thus, zVAD-fmk represents a promising therapeutic agent to reduce SM injury.***

Plans/Milestones for the Next Quarter

Subsequent studies would now utilize additional caspase inhibitors to determine their effectiveness in reducing SM pathology and apoptosis

7. ACCOMPLISHMENT OF TASKS

KEY RESEARCH ACCOMPLISHMENTS PHASE I AND PHASE II

- Discovery that SM induces keratinocyte cell death in large part via a CaM-dependent apoptosis.
- Blocking vesication by inhibiting these pathways in animal models
- Testing of dominant-negative caspase-–9 retroviral vectors to further determine the relative contributions of the death receptor and mitochondrial pathways of SM apoptosis, and to design inhibitors based on these experiments to inhibit SM toxicity and vesication.
- **Successful use of pharmacological inhibitors of caspases and death receptors to block the SM apoptotic response – PHASE II**

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8. CHRONOLOGICAL BIBLIOGRAPHY AND PERSONNEL

Publications (2002-2006):

Publications (peer-reviewed):

1. Simbulan-Rosenthal C. M., Ray R., Benton B., Soeda E., Daher A., Anderson D., Smith W.J, **Rosenthal D. S**. Calmodulin mediates sulfur mustard toxicity in human keratinocytes. *Toxicology*, Oct 3; **227**(1-2): 21-35 (2006). Epub 2006 Jul 13. PMID: 16935404
2. Simbulan-Rosenthal CM, Daher A, Trabosh V, Chen WC, Gerstel D, Soeda E, **Rosenthal DS**. Id3 induces a caspase-3- and -9-dependent apoptosis and mediates UVB sensitization of HPV16 E6/7 immortalized human keratinocytes. *Oncogene,* Jun 22;25(26):3649-60 (2006). Epub 2006 Jan 30. PMID: 16449966
3. Daher, A., Simbulan-Rosenthal, C. M., and **Rosenthal, D. S**. Apoptosis induced by ultraviolet B in HPV-immortalized human keratinocytes requires caspase-9 and is death-receptor independent. *Experimental Dermatology*, **15**: 23-34 (2006).
4. Simbulan-Rosenthal, C. M., Trabosh, V., Velarde, A., Chou, F. P., Daher, A., Tenzin, F., Tokino, T. and **Rosenthal, D. S.** Id2 Protein is selectively upregulated by UVB in primary, but not in immortalized human keratinocytes and inhibits differentiation. *Oncogene*, **24,** 5443-5458 (2005)
5. **Rosenthal, D. S.,** Velena, A., Chou, F.P., Schlegel, R., Ray, R., Benton, B., Anderson, D., Smith, W. J., and Simbulan-Rosenthal, C.M. Expression of dominant-negative Fas-associated death domain blocks human keratinocyte apoptosis and vesication induced by sulfur mustard. *J Biol Chem*. **278**:8531-8540 (2003).
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7. Simbulan-Rosenthal, C. M., Velena, A., Veldman, T., Schlegel, R., and **Rosenthal, D. S.** HPV E6/7 immortalization sensitizes human keratinocytes to UVB by altering the pathway from caspase-8 to caspase-9-dependent apoptosis. *J. Biol. Chem.* **277**:24709-16 (2002).
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Chapters

1. **Rosenthal D. S**., Soeda, E., Daher, A., Ray, R., Benton B., Anderson, D., Smith , W. J. and Simbulan-Rosenthal, C. M. Characterization and Modulation of Proteins Involved in Sulfur Mustard Toxicity and Vesication in Human Keratinocytes and Grafted Human Skin. In *Journal of Burns and Wounds* (Stephen M. Milner, Ed.) In press (2007).
2. Simbulan-Rosenthal, C. M., **Rosenthal, D. S.,** Haddad, B., Ly, D., Zhang, J., and Smulson, M. E. Involvement of PARP-1 and Poly(ADP-ribosyl)ation in the Maintenance of Genomic Stability. In *PARP as a therapeutic target* (Zhang, J. (ed.)), 39-58 (2002).

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1. Simbulan-Rosenthal, C., Bazar, L., Chrikjian, J., and **Rosenthal, D. S.** Detection of high risk oncogenic HPV strains using mismatch repair enzymes. American Association for Cancer Research 96st Annual Meeting, Anaheim, CA (2005).

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4. Ahmad, D., Simbulan-Rosenthal, C., and **Rosenthal, D. S.** Immortalization sensitizes keratinocytes to UVB-induced apoptosis mediated by Id3. Cellular Senescence and Cell Death, Keystone, CO (2005).

5. Chou F., Simbulan-Rosenthal, C., and Rosenthal, D. S. Artemesinin selectively induces apoptosis in tumorigenic human ectocervical and mammary epithelial cells. Cellular Senescence and Cell Death, Keystone, CO (2005).

6. **Rosenthal, D. S.** , Ray, R., Benton, B., Anderson, D., Smith, W., and Simbulan-Rosenthal, C., Characterization and Modulation of Proteins Involved in Sulfur Mustard Vesication. Biosciences 2004 Meeting, Huntvalley, MD (2004).

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9. Smulson, M., Simbulan-Rosenthal, C., Liu, W.F., Velena, A., Anderson, D., Benton, B., Wang, Z-Q., Smith, W., Ray, R., and **Rosenthal, D. S.** PARP determines the mode of cell death in skin fibroblasts, but not keratinocytes, exposed to sulfur mustard. Biosciences 2002 Meeting, Huntvalley, MD (2002)

Personnel

Dean S. Rosenthal, Ph.D.

Cynthia Simbulan-Rosenthal, Ph.D.

Stephanie Garback, Research Technician

Emiko Soeda, Post-Doctoral Fellow

Ahmad Daher, Graduate Student

Attachment J.2.

Quarterly Report Format

1. Contract No DAMD17-00-C-0026 2. Report Date Nov. 13, 2006

3. Reporting period from May 1 , 2005 to July, 31, 2005

4. PI ROSENTHAL, DEAN S., Ph.D. Telephone No. (202) 687- 1056

6. Institution Georgetown University School of Medicine

7. Project Title: Characterization and Modulation of Proteins Involved in Sulfur Mustard Vesication

8. Current staff, with percent effort of each on project.

Dean S. Rosenthal, Ph.D.	30 %	Stephanie Garback (Res. Tech)	100 %
Cynthia Simbulan-Rosenthal, Ph.D.	30 %	Ahmad Daher (Ph.D student)	100 %

9. Contract expenditures to date (as applicable):

	This Qtr/Cumulative		This Qtr/Cumulative
Personnel	$ / $	Travel	0 / $
Fringe Benefits	$	Equipment	0 / 0
Supplies	$ /	Other	$ / $

	This Qtr/Cumulative
Subtotal	$ / $
Indirect Costs	$ $
Fee	0 / 0
Total:	$ / $

10. Comments on administrative and logistical matters.

11. Use additional page(s), as necessary, to describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract. Explain deviations where this isn't possible. Include data where possible.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Attachment J.2.

Quarterly Report Format

1. Contract No DAMD17-00-C-0026 2. Report Date Nov. 13, 2006

3. Reporting period from Aug.1 , 2005 to Oct. 31, 2005

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6. Institution Georgetown University School of Medicine

7. Project Title: Characterization and Modulation of Proteins Involved in Sulfur Mustard Vesication

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		Ahmad Daher (Ph.D student)	100 %
Cynthia Simbulan-Rosenthal, Ph.D.	30 %	Emiko Soeda (Postdoc fellow)	100 %

9. Contract expenditures to date (as applicable):

	This Qtr/Cumulative		This Qtr/Cumulative
Personnel	$ /	Travel	/ $
Fringe Benefits	$ $	Equipment	/
Supplies	/	Other	/ $

	This Qtr/Cumulative
Subtotal	$ / $
Indirect Costs	$ $
Fee	0 / 0
Total:	$ / $

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Personnel		Travel	0 /
Fringe Benefits	$	Equipment	0 / 0
Supplies	$ / $	Other	/

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Subtotal	$ /
Indirect Costs	$
Fee	0 / 0
Total:	$ / $

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12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Attachment J.2.

Quarterly Report Format

1. Contract No DAMD17-00-C-0026 2. Report Date Nov. 13, 2006

3. Reporting period from Feb 1 , 2006 to April 30, 2006

4. PI ROSENTHAL, DEAN S., Ph.D. Telephone No. (202) 687- 1056

6. Institution Georgetown University School of Medicine

7. Project Title: Characterization and Modulation of Proteins Involved in Sulfur Mustard Vesication

8. Current staff, with percent effort of each on project.

Dean S. Rosenthal, Ph.D.	10 %	Ahmad Daher (Ph.D student)	35 %
Cynthia Simbulan-Rosenthal, Ph.D.	30 %	Emiko Soeda (Postdoc fellow)	35 %

9. Contract expenditures to date (as applicable):

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Personnel	$ /	Travel	0 / $
Fringe Benefits	$	Equipment	0 / 0
Supplies	$ /	Other	/

	This Qtr/Cumulative
Subtotal	$ / $
Indirect Costs	$ / $
Fee	/ 0
Total:	$ / $

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12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

U.S. Army Medical Research and Materiel Command Animal Use Report

Facility Name: Georgetown University
Address: 3900 Reservoir Rd. NW
Washington DC 20057

Principal Investigator: _____ (Signature)

Principal Investigator: Rosenthal, Dean S., Ph.D. (Typed/Printed Name)

Award Number: DAMD 17-00-C-0026

This Report is for Fiscal Year (01 October - 30 September): 1 May 05- 30 April 06

AAALAC* Accreditation Status (circle one): (Full) Provisional Not Accredited

Date of Last USDA Inspection: July 10, 2002 USDA Registration Number: 10-R-0004

Definitions of Column Headings on Back of Form					
A. Animal	B. Number of animals purchased, bred, or housed but not yet used	C. Number of animals used involving no pain or distress	D. Number of animals used in which appropriate anesthetic, analgesic, or tranquilizing drugs were used to alleviate pain	E. Number of animals used in which pain or distress was not alleviated	F. Total Number of Animals (Columns C+D+E)
Dogs					
Cats					
Guinea Pigs					
Hamsters					
Rabbits					
Non-human Primates					
Sheep					
Pigs					
Goats					
Horses					
Mice	0	0	180	0	180
Rats					
Fish					
List Others:					

*AAALAC - Association for the Assessment and Accreditation of Laboratory Animal Care